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Biochemistry

Host cell-mediated selection of a mutant influenza A virus that has lost a complex oligosaccharide from the tip of the hemagglutinin

(determination of oligosaccharide chain number and type/determination of HA_1 amino acid sequence/endo-β-*N*-acetylglucosaminidase F and H/unusual glycosylation site sequence)

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ABSTRACT **During serial passage in Madin–Darby bovine kidney (MDBK) cells, a substrain of influenza virus A/WSN is lost from the population and is replaced by a mutant virus with altered host cell binding properties. This selection does not occur during growth in chicken embryo fibroblasts (CEF). It occurs during growth in MDBK cells because the parental virus produced by these cells has a dramatically reduced affinity for cellular receptors [Crecelius, D. M., Deom, C. M. & Schulze, I. T. (1984) *Virology* 139, 164–177]. We have now compared the hemagglutinin (HA) subunits, HA_1 and HA_2, of the parent and mutant viruses by $NaDodSO_4$/PAGE and have found that when the viruses are grown in either host cell the HA_1 subunit of the mutant is smaller than that of the parent virus. The nonglycosylated HAs, made in the presence of tunicamycin, have the same apparent molecular weight, indicating that the HA_1 subunit of the mutant virus contains less carbohydrate than that of the parent. This reduction in carbohydrate content was observed with 11 independently derived mutants that had been selected by growth in MDBK cells. The nucleotide sequence of the HA gene of the parent and mutant viruses indicates that there are five potential glycosylation sites on the parent HA_1 subunit and four on the mutant and that the mutation responsible for this difference is a single base change that eliminates the glycosylation site at amino acid 125 of the parent HA_1 subunit. Treatment of the parent and mutant HAs from both cell sources with endo-β-*N*-acetylglucosaminidases F and H showed that the HA_1 of the parent virus has four complex and one high-mannose oligosaccharides, whereas that of the mutant virus has three complex and one high-mannose oligosaccharides. Thus, all of the potential sites on both HA_1 subunits are glycosylated. We conclude that the oligosaccharide attached to amino acid 125 of the parent HA by MDBK cells can reduce the affinity of the virus for cellular receptors and that the mutant virus has a higher affinity than the parent because the mutant HA is not glycosylated at that site. Since amino acid 125 of the parent HA is glycosylated by both CEF and MDBK cells, we further conclude that the host-determined structure of the oligosaccharide at that site affects the affinity of the parent virus for cellular receptors and, thereby, determines whether the mutant virus will have a growth advantage.**

It has been found that the growth of influenza virus in certain cells can dramatically reduce their affinity for cellular receptors and that mutation in the hemagglutinin (HA) can overcome this effect and can give the mutant virus a selective advantage during growth in those cells (1). This mutation also makes the mutant virus more resistant than the parental virus to neutralization by certain monoclonal antibodies (2). These observations were made by comparing the biological properties of a spontaneous mutant of the WSN strain of influenza virus with those of the parental virus. When grown in chicken embryo fibroblasts (CEF), the two viruses display the same affinity for receptors on CEF cells. When grown in Madin–Darby bovine kidney (MDBK) cells, the affinity of the parental virus for cellular receptors is greatly reduced whereas the affinity of the mutant virus is like that of virus grown in CEF (1). As a consequence of these binding properties, the parent and mutant viruses grow equally well during serial passage in CEF whereas in MDBK cells the mutant virus rapidly replaces the parent. Thus, the host cell type in which the virus is grown determines whether selection of the mutant virus occurs.

Analysis of the HA glycopeptides of the parent virus obtained from both cells has indicated that this selection is most likely a consequence of the bulky oligosaccharides attached to the HA by MDBK cells (3). Our previous work has, however, not provided information on the mechanism by which a mutation in the HA gene overcomes this effect. To this end we have compared the molecular properties of the parent and mutant HAs, using endoglycosidase digestion, serial lectin affinity chromatography, and nucleic acid sequence analysis to determine the number, type, and location of the oligosaccharides on these glycoproteins. We report that the variant HA lacks a complex oligosaccharide that is on the parent HA at amino acid 125 of the HA_1 subunit and that it is the loss of this oligosaccharide that gives the mutant a selective advantage during growth in some hosts. Moreover, we conclude that it is not in itself the addition of an oligosaccharide at this position, but rather the composition of the oligosaccharide as determined by the host cell that can affect the ability of the parental virus to bind to cellular receptors.

MATERIALS AND METHODS

Cell and Virus Growth. MDBK cells and primary CEF were grown as described (1). Stocks of the parent and mutant strains of influenza A/WSN (H1N1) virus, grown in CEF, were prepared as described (2).

Growth, Radioactive Labeling, and Purification of Viruses. Virus for specific experiments was grown in either MDBK or CEF cells and purified as described (1, 4). Radioactive viruses were prepared by the addition of [2-^{3}H]mannose (15 Ci/mmol, 1 Ci = 37 GBq, Amersham) at 2 μCi/ml or [^{35}S]methionine (1400 Ci/mmol, Amersham) at 5 μCi/ml to Dulbecco's modified Eagle's medium (DMEM) containing

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Abbreviations: CEF, chicken embryo fibroblasts; HA, hemagglutinin; endo F, endo-β-*N*-acetylglucosaminidase F; endo H, endo-β-*N*-acetylglucosaminidase H.

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2% (vol/vol) fetal calf serum (MEM–2%) at the time of infection. Purified virus was iodinated with ^{125}I using the chloramine-T procedure (5).

Radioactive Labeling of Glycosylated and Unglycosylated HA in Infected Cells. Confluent cell monolayers were infected at 15 plaque-forming units per cell and incubated at 37°C in MEM–2%. At 6 hr after infection, the infected cells were pulse-labeled for 1 hr with [^{35}S]methionine at 50 μCi/ml in MEM containing one-fourth the normal concentration of methionine and 2% (vol/vol) dialyzed fetal calf serum or with [^{14}C]glucosamine (30–40 Ci/mmol, ICN) at 12.5 mCi/ml in MEM–2%. Following a 15-min chase at 37°C in MEM–2%, the cells were washed three times with ice-cold phosphate-buffered saline (6.5 mM KH_2PO_4, 13.5 mM Na_2HPO_4, and 0.1 M NaCl, pH 7.0) and lysed during a 30-min incubation at 4°C in 10 mM Tris·HCl (pH 7.0), 0.15 M NaCl, 0.5% Triton X-100, and 1% sodium deoxycholate. Nuclei were removed by centrifugation at 12,000 × *g* for 5 min, and the supernatant fraction was made 0.1% $NaDodSO_4$. When tunicamycin (Calbiochem-Behring) was used, it was added to a concentration of 2 μg/ml at 3 hr after infection and was present throughout the pulse and chase periods.

Radioimmunoprecipitation. Radioactive HA polypeptides from infected cell lysates and purified viruses were immunoprecipitated as described (6) using anti-HA monoclonal antibodies obtained from Walter Gerhard (Wistar Institute, Philadelphia, PA).

Endo-β-*N*-acetylglucosaminidase F and H Analysis. Immunoprecipitated ^{125}I-labeled HA polypeptides (80,000–102,000 cpm) were released from Pansorbin (Calbiochem-Behring) by heating for 3 min at 100°C in 75 mM sodium phosphate (pH 6.1), 0.6% $NaDodSO_4$, and 1% 2-mercaptoethanol. Two volumes of 75 mM sodium phosphate (pH 6.1) containing 75 mM EDTA were added and Nonidet P-40 was added to a final concentration of 1%. Endo-β-*N*-acetylglucosaminidase F (endo F) (New England Nuclear), 0.8 unit, was added to the reaction mixture (approximately 260 μl) and incubated at 37°C. At intervals, 35-μl aliquots were removed, 18 μl of 3× $NaDodSO_4$/PAGE sample buffer (7) were added, and the samples were heated at 100°C for 3 min to terminate the enzyme reaction. For endo-β-*N*-acetylglucosaminidase H (endo H) analysis, 5,000–15,000 cpm of immunoprecipitated ^{125}I-labeled HA in 75 mM sodium phosphate (pH 6.1) containing 0.6% $NaDodSO_4$ was diluted with 3 volumes of 100 mM sodium citrate (pH 5.5), 2-mercaptoethanol was added to a concentration of 1%, and the samples were heated for 3 min at 100°C. An equal volume of 100 mM sodium citrate (pH 5.5) containing 2 mM phenylmethylsulfonyl fluoride was added, and the samples (40 μl) were incubated with 0.5 unit of endo H (Miles Scientific, Naperville, IL) at 37°C for 18 hr. Enzyme reactions were terminated as described above for endo F treatment.

$NaDodSO_4$/PAGE. Electrophoresis was performed on polyacrylamide slab gels as described (7). Gels containing ^{35}S- or ^{125}I-labeled proteins were autoradiographed, and those containing radioactive sugars were fluorographed.

Preparation and Fractionation of Glycopeptides. The HA_1 and HA_2 subunits were isolated, digested with pronase, and analyzed by serial lectin affinity chromatography as described (3).

Nucleotide Sequence Analysis. Virion RNA was prepared from purified parent and mutant virus as described (8). The sequence of the HA_1 region of the *HA* gene of each virus was determined by the dideoxy-chain termination procedure (9) as modified by Caton *et al.* (10).

RESULTS

The two HA variants described here grow equally well in CEF, whereas in MDBK cells the mutant virus has a selective advantage that leads to it representing a significant fraction of the progeny after about three serial virus passages (11). This mutation is readily detected by plaque morphology on the MDBK cells; the parent virus produces small plaques with fuzzy edges and the mutant virus produces large, clear plaques. Using this distinction in plaque type, we have isolated mutant virus from plaque-purified parental virus following passage of parental virus in MDBK cells and have compared the molecular properties and the nucleotide sequence of the two viruses within a few passages of isolating the mutant. Plaque morphology was used to confirm the genotype of the viruses grown in both host cells, allowing host determined modification of the viral HA and host-dependent selection of a genetic variant to be distinguished.

Size and Carbohydrate Content of the Parental and Mutant Hemagglutinins. As shown in Fig. 1, a difference in the size of the HAs of the two viruses was observed when virus obtained from both MDBK cells and CEF was analyzed by $NaDodSO_4$/PAGE. Both the uncleaved HA and the HA_1 subunit of the mutant virus grown in CEF and in MDBK cells (lanes 1 and 3) migrated faster than the corresponding parental glycoproteins (lanes 2 and 4). No difference was detected in the mobility of the HA_2 subunits of the two viruses from either cell. When [^{35}S]methionine was used to label the viruses, the nonglycosylated virion proteins (polymerase proteins, nucleoprotein, and matrix protein) from the two viruses were identical in mobility (data not shown), as were the neuraminidase glycoproteins from CEF-grown viruses (Fig. 1, lanes 1 and 2). The neuraminidases from viruses grown in the MDBK cells were not well enough separated from the HA_1 subunits to be detected. Differences in the mobility of the HA when grown in different hosts have been observed (12, 13) and are due to differences in the oligosaccharides attached to the HA by the particular host cells (3, 14, 15).

The difference in the migration rate of the parental and mutant HA glycoproteins obtained from the same host could be due to a difference in carbohydrate content, polypeptide chain size, or both. To distinguish among these possibilities, infected cells were pulse-labeled with [^{35}S]methionine in the absence and presence of tunicamycin, and the immunoprecipitated HA proteins were analyzed by $NaDodSO_4$/PAGE. Since this procedure analyzed cell-associated HA rather than that assembled into virus, virtually all of the labeled HA was in the uncleaved form. The glycosylated HA again varied in mobility depending on the virus and the cell source (Fig. 2*A*, lanes 2–5), while the unglycosylated HA proteins were identical in mobility (lanes 6–9). The second most rapidly migrating bands seen in lanes 6 and 7 were frequently obtained from CEF extracts but varied in amounts from

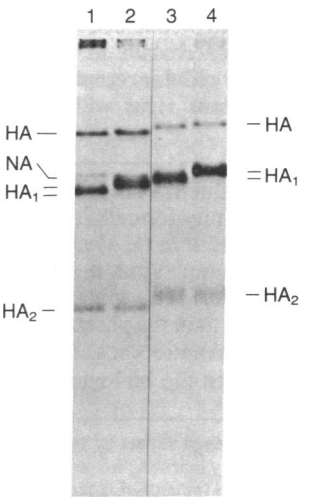

FIG. 1. $NaDodSO_4$/PAGE analysis of [^3H]glucosamine-labeled mutant and parent viruses obtained from CEF and MDBK cells. A 17.5% acrylamide/4 M urea gel was used. Lanes: 1, CEF-grown mutant virus; 2, CEF-grown parental virus; 3, MDBK-grown mutant virus; 4, MDBK-grown parental virus.

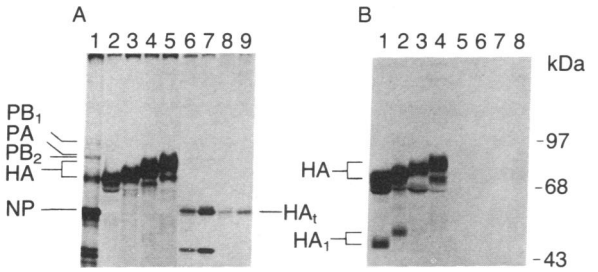

FIG. 2. NaDodSO$_4$/PAGE analysis of mutant and parental HA proteins synthesized in the absence and presence of tunicamycin. CEF and MDBK cells infected with parental or mutant viruses were pulse-labeled and samples were analyzed on 10% polyacrylamide gels. All samples except that in *A*, lane 1, were immunoprecipitated with anti-HA antibodies. (*A*) [^{35}S]methionine-labeled cells. Lanes: 1, mutant virus-infected CEF lysate; 2, mutant virus-infected CEF; 3, parental virus-infected CEF; 4, mutant virus-infected MDBK; 5, parental virus-infected MDBK; 6, mutant virus-infected CEF plus tunicamycin; 7, parental virus-infected CEF plus tunicamycin; 8, mutant virus-infected MDBK plus tunicamycin; 9, parental virus-infected MDBK plus tunicamycin. (*B*) [^{14}C]glucosamine-labeled cells. Lanes 1–8 same as lanes 2–9 in *A*.

preparation to preparation. They represent partial degradation products of the unglycosylated parental and mutant HAs. N-linked glycosylation was completely inhibited by the amount of tunicamycin added (Fig. 2*B*). The data indicate that the variations in mobility of the glycosylated HAs were due to differences in carbohydrate content and not to differences in the size of the HA polypeptides.

Correlation Between Biological Properties and the Size of the Hemagglutinins. A difference in affinity of the mutant and parent virus HAs from MDBK cells for cellular receptors can be readily demonstrated by carrying out hemagglutination reactions in the presence of calf serum at 4°C and at 37°C (1). Whereas mutant virus from MDBK cells agglutinates erythrocytes equally well at the two temperatures, the parent virus from MDBK cells agglutinates erythrocytes at 4°C but not at 37°C. We have used these properties of the two HAs to test the correlation between the size of the HA$_1$ subunits and the biological properties of individual mutant viral isolates. Ten parental virus plaques were isolated and grown in CEF cells, and each stock was serially passaged in MDBK cells. Mutant virus plaques that appeared by the third passage were picked and stocks of each cloned virus were prepared in CEF. The plaque morphology of each clone was confirmed, the hemagglutinating activity of each virus obtained from MDBK cells was measured at 4°C and at 37°C, and the sizes of the glycosylated and unglycosylated HA were determined. As shown in Table 1, all 10 mutant virus clones were identical to the prototype mutant virus (designated C$_0$) and distinct from

Table 1. Phenotypic properties of mutants selected by growing parental virus in MDBK cells

Virus	Plaque type on MDBK cells	HAU* (4°C/37°C)	$M_r \times 10^{-3}$† HA	HA$_1$	HA$_2$	HA$_{TM}$
Parent	F	128–256	75	52	25	63
Mutant 0	C$_0$	1	73	49	25	63
Mutant 1–10	C	0.5–2.0	73	49	25	63

F, parent; C, mutant; TM, tunicamycin.
*Ratios determined on MDBK-grown virus as described (1); HAU, hemagglutinating units.
†M_r determined by NaDodSO$_4$/PAGE of [^{35}S]methionine-labeled CEF-grown HA proteins that had been immunoprecipitated with anti-HA monoclonal antibodies.

the parental virus. All of the mutants had hypoglycosylated HA$_1$ subunits. This correlation between the amount of carbohydrate on the HA$_1$ subunit and the ability of the virus to grow well in MDBK cells and agglutinate erythrocytes strongly suggested that the decrease in carbohydrate content that accompanied the transition from parent to mutant was responsible for the increased affinity of the mutant virus for MDBK cell receptors.

Identification of Sequence Changes Responsible for the Differences in Oligosaccharide Content. Direct sequence analysis of the entire HA$_1$ region of the HA gene of both viruses was performed to identify the mutation responsible for the reduced oligosaccharide content of the mutant HA and to determine the number and the location of the potential glycosylation sites (Asn-Xaa-Ser/Thr sequences) on the two HAs. The HA$_1$ regions of the parental and mutant virus HA genes were found to be identical in sequence except at nucleotide 456. The mutant virus has a guanosine residue instead of an adenosine residue at position 456, which changes amino acid 125 from asparagine to aspartic acid and abolishes a glycosylation site that is present in the parent virus. Four additional potential glycosylation sites, at asparagine residues 10 or 11, 56, 179, and 268, were found on both the parent and mutant HAs. Thus, the HA$_1$ subunit of the parent virus has five potential glycosylation sites, whereas that of the mutant has only four.

The nucleotide sequence of the HA$_1$ region of the parental and mutant viruses differs from that described for the WSN strain (16) at eight positions; the parental and mutant substrains both have a cytidine at nucleotide 141, a cytidine at 191, an adenosine at 508, an adenosine at 621, an adenosine at 626, a uridine at 629, a guanosine at 742, and a guanosine at 933 [residues are numbered according to Hiti *et al.* (16)]. These nucleotide differences indicate that both substrains studied here differ from the WSN strain previously sequenced (16) at amino acid residues 20, 36, 142, 179, 220, and 284. The mutant substrain also differs at residue 125 as indicated above. The remaining two nucleotide differences (at residues 626 and 629) do not alter the amino acid sequence. The adenosine at residue 621 in both the parent and mutant viruses puts asparagine into the amino acid sequence at position 179 and generates a potential glycosylation site where none was observed in the strain sequenced by Hiti *et al.* (16). These sequence differences have presumably occurred during independent laboratory passage of a single virus isolate, as has been noted (10).

Number and Types of Oligosaccharides on the Parent and Mutant Hemagglutinins. Two enzymes, endo H and endo F, were used to determine the number and the types of oligosaccharides present on the two HAs. Both of these enzymes cleave N-linked oligosaccharides between the two *N*-acetylglucosamine residues proximal to the N-linkage. Endo H cleaves only high-mannose oligosaccharides (17), while endo F cleaves both high-mannose and complex oligosaccharides (18). Controlled digestion of the two HAs from both host cells followed by NaDodSO$_4$/PAGE was used to reveal the number and the types of oligosaccharides on these HAs. Treatment with endo F of ^{125}I-labeled HAs from virus grown in CEF resulted in the stepwise removal of five oligosaccharides from the parent virus HA$_1$ subunit (Fig. 3*A*, lanes 2–8) and four oligosaccharides from the mutant virus HA$_1$ subunit (Fig. 3*B*, lanes 2–8). Prolonged treatment with endo H reduced the size of each HA$_1$ subunit by approximately one oligosaccharide, as judged from the mobility of the first endo F intermediate (Fig. 3 *A* and *B*, lane 1 vs. lane 3 or 4), indicating that each HA$_1$ subunit contains only one high-mannose oligosaccharide (see also Fig. 3*C*, lanes 1–4). The data indicate that the parent HA$_1$ subunit contains a complex oligosaccharide that is not present on the HA$_1$ of the mutant.

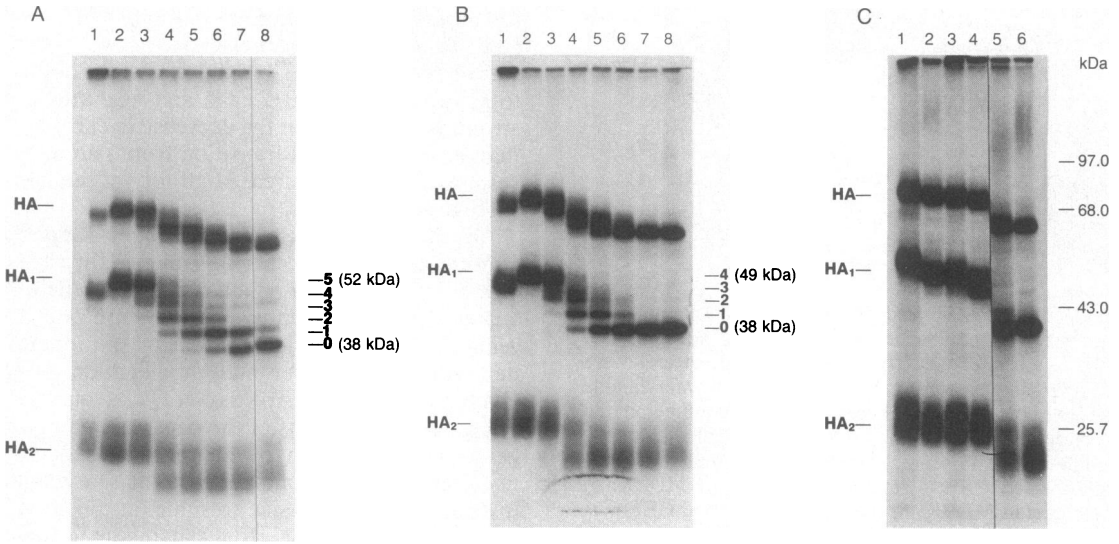

FIG. 3. NaDodSO$_4$/PAGE analysis of ^{125}I-labeled HA polypeptides following treatment with endo H or endo F. Immunoprecipitated ^{125}I-labeled HA from parental and mutant viruses grown in CEF cells was treated with endo H or endo F and analyzed on 10% polyacrylamide gels. (*A*) HA proteins from parental virus. Lane 1: treated with endo H for 18 hr. Lanes 2–8: treated with endo F for 0, 1, 5, 10, 20, and 80 min and 18 hr, respectively. (*B*) HA proteins from mutant virus. Samples were treated as described in *A*. (*C*) Endo H- and endo F-treated HA proteins from parental and mutant viruses. Lanes: 1 and 2, parental and mutant virus HA proteins, respectively; 3 and 4, parental and mutant virus HA proteins treated for 18 hr with endo H; 5 and 6, parental and mutant virus HA proteins treated for 18 hr with endo F. The numbers to the right of each gel indicate the number of oligosaccharides remaining attached to the HA$_1$ subunits and the molecular sizes of the intact and deglycosylated proteins.

Fig. 3*C* also shows that the end products of endo F digestion of the two HAs are identical in size. That these polypeptides were completely deglycosylated was shown by prolonged treatment of [^{14}C]glucosamine-labeled parental and mutant HAs with endo F. This treatment removed all of the labeled carbohydrate but left Coomassie blue-stained bands with mobilities like those observed with the ^{125}I-labeled endo F end products shown in Fig. 3*C*, lanes 5 and 6. Without any carbohydrate the HA$_1$ subunit had an apparent molecular weight of 38,000, and the uncleaved HA corresponded in size to the unglycosylated molecule synthesized in the presence of tunicamycin (M_r 63,000).

Lastly, Fig. 3 shows that the HA$_2$ subunits of both HAs are resistant to endo H but sensitive to endo F. This confirms our observation that no high-mannose oligosaccharides could be detected on the HA$_2$ subunits by chromatography on Con A-Sepharose (3).

Experiments identical to those presented in Fig. 3 were carried out with MDBK grown viruses, and the same results were obtained, except that more diffuse bands were observed, and the rate of deglycosylation was slower. The completely deglycosylated molecules had the same molecular weights as those shown in Fig. 3. The results indicate that all potential sites on both the parent and mutant HA$_1$ subunits are glycosylated by both host cells.

Characterization of the Oligosaccharides on the Parental and Mutant Hemagglutinins by Serial Lectin Affinity Chromatography. To determine whether there were differences in the composition of the complex oligosaccharides, as well as to confirm the difference in the number of carbohydrate chains, the oligosaccharides on the HAs were characterized by serial lectin affinity chromatography. Differences between the HA$_1$ subunits of the two viruses were detected with the first lectin column of the series Con A-Sepharose, which separates glycopeptides with N-linked oligosaccharides into three fractions containing tri- and tetraantennary complex, biantennary complex, or high-mannose oligosaccharides (19). As shown in Table 2, the ratio of complex to high-mannose oligosaccharides was higher for the parent HA$_1$ subunit than for the mutant, and much of the extra complex carbohydrate on the parent molecule was biantennary in structure. All subsequent elution profiles of the HA$_1$ subunit glycopeptides were the same for the two viruses. The HA$_2$ subunits from the two viruses gave identical elution profiles on all columns (data not shown).

Table 2 also shows that the distribution of mannose between total complex and high-mannose oligosaccharides was determined by the viruses and not by the host cells. This distribution was used to calculate the ratio of complex to high-mannose oligosaccharides present on each HA$_1$ subunit. All N-linked complex carbohydrates contain three mannose residues per oligosaccharide. Assuming that high-mannose oligosaccharides contain an average of eight to nine mannose residues per molecule, the data are consistent with the finding (see Fig. 3) that there are four complex oligosaccharides per high-mannose oligosaccharide on the parental HA$_1$ subunits. With the mutant virus this ratio is three to one. The data support the conclusion that the mutant virus HA$_1$ subunit has one less complex oligosaccharide than the parent.

Table 2. Con A-Sepharose affinity analysis of [^{3}H]mannose-labeled glycopeptides derived from HA$_1$ subunits of parent and mutant viruses

		Glycopeptides, %				Ratio, complex (I + II)/ high mannose
		Complex			High-	
Virus	Cells	I	II	I + II	mannose	
Parent	MDBK	50	14	64	36	1.78
Mutant	MDBK	48*	3*	51	49	1.04
Parent	CEF	39	23	62	38	1.63
Mutant	CEF	38	14	52	48	1.08

Pronase digests of [^{3}H]mannose-labeled HA$_1$ subunits were fractionated into tri- and tetraantennary complex (I), biantennary complex (II), and high-mannose glycopeptides by chromatography on Con A-Sepharose. Values indicate the distribution of [^{3}H]mannose-labeled glycopeptides expressed as percentages of recovered counts. *In a duplicate experiment, values were 46% and 6% for fractions I and II, respectively. All other values varied 2% or less.

DISCUSSION

There have been a number of reports correlating sequence changes in the HA_1 subunit with changes in the ability of both influenza A and B virus strains to grow in cells from avian versus mammalian species (20–25). In some cases (22, 25), these changes involve base substitutions that result in the loss of a potential oligosaccharide attachment site from the tip of the HA_1 subunit. Although the host cell binding properties and the number of oligosaccharides attached to the HA had not been determined, these observations prompted the proposal that a mutation that removes an oligosaccharide from the vicinity of the receptor binding site might be expected to increase the capacity of the virus to bind to certain cells (22, 25). The information presented here indicates that the cells in which the parent and mutant viruses are grown determines whether this is indeed the case. Having demonstrated clear differences in the host cell binding properties of parent and mutant viruses grown in MDBK cells (1), we show here that the parent HA_1 subunit has an asparagine-linked oligosaccharide at amino acid 125 whereas the mutant does not and that there are no other differences in the amino acid sequence of the two HA_1 subunits. In addition, our analysis of 11 mutants, independently selected from parental virus by growth in MDBK cells, has indicated that the ability of the virus to grow well in MDBK cells is consistently accompanied by the loss of an oligosaccharide from the HA_1 subunit.

Based on the sequence and the crystalline structure of the H3 trimer (26), amino acid 125 of H1 strains is equivalent to amino acid 129 in the H3 sequence (27) and is in the loop between strands 1 and 2 of the β-sheet structure on the globular head of the HA monomer. It is in the Sa antigenic site of the H1 HA (10), in the vicinity of the receptor binding pocket (26, 28). We, therefore, conclude that the loss of an MDBK cell-synthesized oligosaccharide from the vicinity of the receptor binding pocket increases the capacity of the virus to bind to host cells.

Our data further indicate that this effect of oligosaccharide removal is not observed when these viruses are grown in CEF. In this case the presence of an oligosaccharide at amino acid 125 has little or no capacity to interfere with the binding of the virus to CEF receptors and loss of this oligosaccharide does not increase the ability of the virus to grow in CEF. We conclude that it is not glycosylation *per se* but rather the composition of the oligosaccharides at amino acid 125 that affects the affinity of the parent virus for cellular receptors.

It is interesting in this regard that the parental virus has two sites on the tip of the HA monomer at which the host cells add oligosaccharides whereas other H1 strains have at most one. The second oligosaccharide is at amino acid 179 (184 in H3) that is in the loop between β-strands 3 and 4 and is also in close proximity to the receptor binding pocket. This glycosylation site is present in both the parent and mutant HAs, but is not present in other H1 or H3 sequences. It is unusual in that it consists of Asn-Pro-Ser, followed by two additional serine residues. This sequence is glycosylated, despite the presence of the proline residue (29, 30), since the number of oligosaccharides attached to the HA_1 subunits equals the number of Asn-Xaa-Ser/Thr sequences found in the proteins and no Asn-Xaa-Cys sequences (31) are present.

The presence of this second attachment site in the vicinity of the receptor binding site raises the possibility that the loss of the oligosaccharide at amino acid 179 instead of the one at amino acid 125 could produce the mutant phenotype. This can be determined by sequencing the remaining 10 mutants that we have isolated. This possibility is suggested by the fact that the WSN strain sequenced by Hiti *et al.* (16) was grown in MDBK cells and contains a potential glycosylation site at amino acid 125 but not at 179.

The information presented here illustrates the interplay of the amino acid sequence of the HA and the host cell glycosylating system in determining the structure of the HA and the ability of this glycoprotein to interact with host cells receptors. It identifies two interrelated but distinguishable processes by which host cells can influence influenza A virus host range. Firstly, the nature of the cell-specified oligosaccharides can affect the binding properties of the HA. Secondly, these host-determined binding properties can promote substrain selection during growth in certain hosts and can, thereby, alter the genetic composition of the progeny population. Lastly, these experiments show that receptor variants of the influenza viruses can be selected by host cells by a process that is mechanistically distinct from that associated with amino acid substitutions at residues in the receptor binding pocket (21). Those observations indicated that host cell selection of influenza virus strains can be driven by differences in the ability of the HAs to bind to the specific sialic acid-galactose linkages found in various animal species (32). The information presented here indicates that selection can also be due to differences in receptor affinity determined by the cell-specified oligosaccharides on the tip of the HA.

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